Title: Insulin resistance due to macrophage recruitment in to the inflamed adipose tissue.
Name of the Candidate: Dipanjan Chattopadhyay
Supervisor: Dr. Sutapa Mukherjee and Professor Samir Bhattacharya
Date of Registration: 28/11/2016

Insulin resistance due to macrophage recruitment in to the inflamed adipose tissue.

Incidence of type 2 diabetes (T2D) is dramatically increasing in the past few decades and currently affecting more than 382 million people and by 2035 this may rise up to 592 million over the globe according to ADA, 2016. T2D is the epidemic disease and not type I diabetes (T1D), among the diabetic patients 95-97% are T2D while only 3-5% are T1D (Zimmet et al., 2001). Primary reason for which T2D occurs is the excess accumulation of lipid. It is the lipid that affects insulin resistance, induces inflammation and finally associates inflammation, immunity to affect loss of insulin sensitivity, a stage which occupies the centre of pathogenesis in T2D (Boden et al., 1999, Lumeng et al., 2011, Rosen et al., 2014). We are now aware that almost all crucial chronic diseases are inflammatory in nature. In this scenario, it is intriguing to note that adipocytes are the major cells in adipose tissue, there are other cells too, but these are the cells which could uptake lipid, store them and metabolize them for energy yield. In addition, these cells are now considered to be the most dynamic cell, a cell with limited space for cytoplasmic organelles could secrete 347 proteins, many of them are associated with the regulation of critical metabolic disorders such as type 2 diabetes, atherosclerosis, cardiac problems, etc(Lehr et al., 2011). Several reports in this direction indicated that excess lipid cause adipose tissue inflammation which is now considered as a major cause for insulin resistance and type 2 diabetes(Glass et al., 2012, Gregor et al., 2011, Johnson et al., 2013). However, how excess lipid cause adipose tissue inflammation that leads to their dysfunction is an interesting problem which still remains unresolved.

Interestingly, recruitment and accumulation of a considerable amount of macrophages in adipose tissue greatly contributes to their inflammation which is due to the polarization of proinflammatory M1 phenotype from anti-inflammatory M2 subtype (Lumeng et al., 2007, Lumeng et al., 2007). One can imagine the peak inflammatory status when both adipocytes and macrophages are cohabitants and releasing inflammatory cytokines which inflicts crucial metabolic disorders. It has been reported that inflamed adipocyte secretes variety of negative signals which augments insulin resistance that leads to T2D. One of such signals is MCP-1(Monocyte chemoattractant protein 1) which is a potent chemoattractant for macrophage

towards adipose tissue (Kanda et al., 2006). One of the previous report (Chatterjee et al., 2013) demonstrated that lipid induced release of an acute phase protein i.e. Fetuin-A (FetA)

from adipocytes could attract macrophage towards adipocytes and cause polarization of macrophages. The exact regulatory mechanism behind the macrophage migration and polarization in the ambiance of these endocrine cells remains yet unclear, primarily because of the lack of identification of signal(s) or factor(s), which appears to be originated from inflamed adipose tissue ambience. In the present proposal, I will address the mechanistic aspects of macrophage contribution to adipose tissue inflammation that ultimately produce insulin resistance.

KNOWLEDGE GAP

Adipose tissue inflammation is currently regarded as the prime factor driving insulin resistance. Nevertheless, the mechanisms are not fully understood. One of the key signalling pathways related to tissue inflammation involves NLRP3 inflammosome (Abderrazak et al., 2015, Jo et al., 2015). The activation of NLRP3 by various obesity related danger signals and its corresponding downstream signalling effects leading to adipose tissue inflammation needs to be investigated.

Infiltration of macrophages and their polarisation from M2 to M1 (Weisberg et al., 2003, Xu et al., 2003, Thomas et al., 2017, Morinaga et al., 2012) severely increases inflammatory burden of adipose tissue which significantly contribute to insulin resistance and thus leads to T2D. Although there are several reports demonstrated the release of negative signals from inflamed adipose tissue like MCP1 and FetA that cause macrophage infiltration and polarisation, the regulation of this polarisation is still a shaded area.

In inflamed adipose tissue both adipocyte and macrophage secrete negative signals which causes insulin resistance. But their individual contribution is still unclear. Besides ,how the negative signals secreted by the inflamed adipose tissue (both adipocyte and macrophage)is related to the impairment of insulin sensitivity is an important subject of investigation where further research is necessary.

There are few reports involving inflammatory signalling pathway in macrophage during hyperlidemic environment (Coenen et al., 2007) but which signalling pathway plays the major role in macrophage inflammation is unknown and needs to be answered. Moreover

how ATM (adipose tissue macrophage) can be modulated to protect against the metabolic effects of obesity is poorly known.

Objectives :

1. To investigate the activation of NLRP3, one of the key inflammatory signalling pathway in adipocytes under hyperlipidemic condition.

2. To determine the interactions between adipocyte and macrophages in inflamed adipose tissue to cause insulin resistance. It is also crucial to elucidate the major molecular signalling pathway involved in macrophage inflammation.

3. To investigate the effect of different plant derived and/or other molecules that can inhibit ATM infiltration and polarisation from M2 to M1 in inflamed adipose tissue and enhance insulin sensitivity

Methodology :

I. Models of study: The study will be conducted in different models as mentioned below

(i) Animals – Adult normal healthy male BALB/c mice would be conditioned with a 12 h light/12 h dark cycle at 23 ± 2°C and relative humidity 55 ± 5% along with access to standard diet *ad libitum*. A subset of control BALB/c mice would be reared on high-fat-diet (HFD) for 12 weeks to render them insulin resistant(Bhattacharya et al., 1976, Pal et al., 2012). Besides, genetically modified strains of mice established as obese and type 2 diabetic models viz. *db/db* mice would also be considered for study. Body fluids and tissues,

visceral adipose tissue would be obtained from the animals. Animal handling, maintenance and experiments would be performed following approval and guidelines prescribed by the Institutional Ethics Committee.

(ii) Primary culture – Adipocytes would be isolated from animal models and cultured *in vitro* following our earlier method.[14] In brief, adipose tissue would be rinsed with sterile 0.9% NaCl and thoroughly washed in HBSS supplemented with 5.5 mM glucose. Adipose tissue would be then digested in HBSS buffer containing 5.5 mM glucose, 5% fatty acid-free BSA, and 3.3 mg ml^{-1} of type II collagenase for 30 min at 37 °C. The digestion mixture would be passed through tissue sieve and adipocytes suspended in serum-free DMEM. Finally the cells would be plated in 6-well culture plates in humidified 5% CO_2 at 37 °C. Macrophages would be isolated from peritoneal fluid and cultured in serum-free RPMI in 6-well culture plates in humidified 5% CO_2 at 37 °C.

(iii) Cell lines – Mice macrophage cell line RAW264.7, mice 3T3-L1 preadipocytes, human THP1 monocytic cell line differentiated before treatment etc. would also be used for study.

I. Treatments: To create an insulin resistant model, the control mice would be fed high-fat-diet (HFD) for 12 weeks as earlier done by us.[4,14] The HFD consists of 32.5% lard, 32.5% corn oil, 20% sucrose, and 15% protein whereas standard diet consists of 57.3% carbohydrate, 18.1% protein, and 4.5% fat. The energy content of standard diet is 15 kJ/g and HFD is 26 kJ/g. All cell culture treatments would be conducted in serum-starved condition. Treatments with palmitate, Fetuin-A, SIRT1 and several inhibitors and other chemicals would be carried out. Various short-interfering siRNA and vector transfections would be performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

II. 2-deoxyglucose uptake: Isolated adipocytes and skeletal muscle cells would be incubated in Krebs-Ringer phosphate buffer supplemented with 0.2% BSA followed by 30 min of incubation in the presence of insulin (100 nM). [^{3}H]-2-

deoxyglucose (0.4 nmol/ml) would be added to each incubation 5 min before the termination of incubation. Cells would then be washed three times with ice-cold Krebs buffer in the presence of 0.3 mM phloretin. The cells would be solubilized with 1% NP-40 and [^{3}H]-2-deoxyglucose would be measured in liquid scintillation counter.

III. **[^{14}C]-Palmitate uptake:** Adipocytes without or with fetuin-A would be incubated with 1 μCi/mol [^{14}C]-Palmitate for 3 h. Cells would be washed three times with ice-cold Krebs-Ringer phospahe buffer. Cells would be solubilized with 1% NP-40 and [^{14}C]-palmitate would be measured in liquid scintillation counter.

IV. **Transwell system of co-culture:** In order to simulate the microenvironment that exists during inflammatory condition, indirect co-culture would be performed *in vitro* using transwell system (Thermo Nunc). Macrophages would be seeded on the bottom whereas adipocytes would be cultured onto the membrane of transwell cell culture inserts. Co-cultures would be treated as required and the media and/or cell lysates would then be finally analyzed for gene/protein expressions (Chatterjee et al., 2012)

V. **Cell migration assay:** Macrophage cell migration assay would be performed in Boyden chamber system (Millipore QCM 24-well colorimetric cell migration assay kit). Addition of fetuin-A, and other chemoattractants and cytokines/chemokines would be done in the lower chamber. After the required period of incubation, the cells would be stained and photographed. The absorbance would be then measured in 96-well microplate reader (Chatterjee et al., 2012)

VI. **ChIP assay:** Chromatin Immunoprecipitation would be performed by using ChIP assay kit (Upstate) in human/mice adipocytes by using anti-NF-κBp65 and species-specific fetuin-A promoter sequence. PCR products would be

resolved in ethidium bromide-stained 1.5% agarose gel, and image captured in Bio-Rad gel documentation system.

VII. **Immunofluorescence:** Paraformaldehyde-fixed cells would be incubated with respective primary antibodies followed by incubation with FITC-conjugated secondary antibody for 2 h. Cells would be mounted in DAPI mounting medium for nuclear staining if required. Immunofluorescence would be seen with a fluorescence microscope (Lumeng et al.,2007).

VIII. **Protein estimation:** The protein content of tissue extracts, cell lysates and media would be determined by the method of Lowry *et al*.

IX. **Immunoblotting:** Protein content (50 μg) from tissue extracts, cell lysates and media would be resolved by SDS-PAGE and then transferred on to PVDF membranes (Millipore) using Semi-Dry Trans-Blot® Apparatus (Bio-Rad Laboratories). The membranes would be then incubated with primary antibodies followed by secondary antibody conjugated to alkaline phosphatase and also horse redox peroxidase. Protein bands would be detected by using BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (nitro blue tetrazolium) and also Luminol.

X. **Reverse-transcription PCR (RT-PCR) and real-time PCR:** Total RNA would be extracted from different incubations using TRI Reagent (Sigma) or RNeasy Lipid Tissue Mini Kit (Qiagen) according to manufacturer's instructions. RT-PCR would be carried out using first–strand cDNA synthesis kit (Fermentas). Gene expression would be further validated by SYBR green based quantitative real-time PCR (Applied Biosystems) by using gene-specific primers. The housekeeping gene, *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) would be simultaneously amplified in separate reactions for correcting the C_t value.

XI. **ELISA:** Various proinflammatory macrophage M1 markers (TNFα, IL-6, IL-1β etc.) and anti-inflammatory macrophage M2 markers (IL-10 etc.), fetuin-A, other signaling molecules would be measured in cell culture media or lysates or tissue extracts by enzyme-linked immunosorbent assay using ELISA kits (Ray Biotech) according to manufacturer's instructions.

XII. **FACS:** M1 macrophages would be labeled using CD11c antibody (BD Pharmingen) or with their isotype control (Santacruz Biotechnology) for 30 min at 4°C. After washing with PBS, the labeled cells would be analyzed by flow cytometry using a BD FACSAria™ II and FACS Diva Software. Similarly, macrophages would be labeled for other cell surface markers.

XIII. **Immunohistochemistry :** The pancreas will be isolated from both the control and HFD groups. The pancreas will be fixed in Bouin's fluid followed by dehydration in ascending grades of alcohol. Paraffin embedded tissues will be cut at appropriate thickness using a Microtome and affixed on glass slides for further observation. The sections will be deparaffinised in xylene and rehydrated with descending grades of alcohol. For immunohistochemical labeling, tissue sections will be sequentially treated with blocking solution (1–2 h), incubated in primary antibodies overnight (4°C), and incubated with appropriate Alexa fluor-conjugated secondary antibodies (1–2 h) the following day. Draq5 (Biostatus Limited) at 1:3000 dilution will be used as a nuclear counterstain. Immunofluorescently labeled M1 and M2 cells will be quantified under high-power magnification with wide field-fluorescence (Lumeng et al., 2007). Macrophage specificity will be confirmed by double labelling with macrophage markers (tomato lectin) and M1 or M2 markers (TNFα, IL-6, MCP-1 and PPARγ, 1L-10 respectively)

XIV. **Statistical Analysis** : Data will be analyzed by one-way analysis of variance (ANOVA) where the F value indicated significance, means will be compared by a post hoc multiple range test. All values will be represented as mean ± SEM taking $P<0.05$ as threshold of significance

Keywords

Insulin resistance, Adipose tissue, Macrophage, Type 2 diabetes, Inflammation, Inflammasome, Polarisation

References:

1. Abderrazak, A., Syrovets, T., Couchie, D., El Hadri, K., Friguet, B., Simmet, T., & Rouis, M. (2015). NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. *Redox biology*, *4*, 296-307.
2. Bhattacharya, S., Das, R. H., & Datta, A. G. (1976). Iodine metabolism in dispersed pharyngeal and head kidney teleostean thyroid cells obtained by continuous trypsinization. *General and comparative endocrinology*, *30*(1), 128-130.
3. Chatterjee, P., Seal, S., Mukherjee, S., Kundu, R., Mukherjee, S., Ray, S., ... & Bhattacharya, S. (2013). Adipocyte fetuin-A contributes to macrophage migration into adipose tissue and polarization of macrophages. *Journal of Biological Chemistry*, *288*(39), 28324-28330.
4. Glass, C. K., & Olefsky, J. M. (2012). Inflammation and lipid signaling in the etiology of insulin resistance. *Cell metabolism*, *15*(5), 635-645.
5. Gregor, M. F., & Hotamisligil, G. S. (2011). Inflammatory mechanisms in obesity. *Annual review of immunology*, *29*, 415-445.
6. Jo, E. K., Kim, J. K., Shin, D. M., & Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & molecular immunology*, *13*(2), 148-159.
7. Johnson, A. M., & Olefsky, J. M. (2013). The origins and drivers of insulin resistance. *Cell*, *152*(4), 673-684.
8. Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K. I., Kitazawa, R., ... & Kasuga, M. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *Journal of Clinical Investigation*, *116*(6), 1494.
9. Lehr, S., Hartwig, S., Lamers, D., Famulla, S., Müller, S., Hanisch, F. G., ... & Sell, H. (2012). Identification and validation of novel adipokines released from primary human adipocytes. *Molecular & cellular proteomics*, *11*(1), M111-010504.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein estimation by Lowry's method. *Journal of Biological Chemistry*, *193*, 265.

11. Lumeng, C. N., & Saltiel, A. R. (2011). Inflammatory links between obesity and metabolic disease. *The Journal of clinical investigation*, *121*(6), 2111.

12. Lumeng, C. N., Bodzin, J. L., & Saltiel, A. R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *Journal of Clinical Investigation*, *117*(1), 175.
13. Lumeng, C. N., DeYoung, S. M., Bodzin, J. L., & Saltiel, A. R. (2007). Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes*, *56*(1), 16-23.
14. Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S., ... & Bhattacharya, S. (2012). Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nature medicine*, *18*(8), 1279-1285.
15. Rosen, E. D., & Spiegelman, B. M. (2014). What we talk about when we talk about fat. *Cell*, *156*(1), 20-44.
16. Santomauro, A. T., Boden, G., Silva, M. E., Rocha, D. M., Santos, R. F., Ursich, M. J., ... & Wajchenberg, B. L. (1999). Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. *Diabetes*, *48*(9), 1836-1841.
17. Thomas, D., & Apovian, C. (2017). Macrophage functions in lean and obese adipose tissue. *Metabolism*.
18. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante Jr, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of clinical investigation*, *112*(12), 1796.
19. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., ... & Chen, H. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of clinical investigation*, *112*(12), 1821.
20. Zimmet, P., Alberti, K. G. M. M., & Shaw, J. (2001). Global and societal implications of the diabetes epidemic. *Nature*, *414*(6865), 782-787.